AR226-0083

PFOS:
A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE FATHEAD MINNOW (*Pimephales promelas*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-102

3M LAB REQUEST NO. U2723

U. S Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1075
and
OECD Guideline 203

AUTHORS:

Kurt R. Drottar
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: December 4, 1998

STUDY COMPLETION DATE: July 14, 1999

AMENDED REPORT DATE: April 26, 2000

Submitted to

3M Corporation
Environmental Laboratory
935 Bush Avenue
St. Paul, Minnesota 55144

Wildlife International Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

000543

AMENDED

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-102

STUDY COMPLETION: July 14, 1999

AMENDED REPORT: April 26, 2000

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984 with the following exceptions:

The test substance was not characterized in accordance with full GLP compliance; however, the characterization was performed according to 3M Standard Operating Procedures and Methods, and all raw data are being maintained in the 3M archives. The test substance is being recharacterized in accordance with GLP.

The stability of the test substance under conditions of storage at the test site was not determined in accordance with Good Laboratory Practice Standards.

STUDY DIRECTOR:

Kurt R. Drottar
Senior Biologist

4/26/00
DATE

SPONSOR APPROVAL:

Sponsor

4/27/00
DATE

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992; and Japan MAFF, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	February 11, 1999	February 11, 1999	February 11, 1999
Test Initiation	February 15, 1999	February 15, 1999	February 16, 1999
Matrix Fortification Preparation	February 15, 1999	February 15, 1999	February 18, 1999
Water Chemistry Measurements	February 17, 1999	February 17, 1999	February 19, 1999
Biological Data and Draft Report	March 17, 1999	March 17, 1999	March 24, 1999
Analytical Data and Draft Report	March 22 – 24, 1999	March 24, 1999	March 24, 1999
Final Report	July 14, 1999	July 14, 1999	July 14, 1999
Amended Report	April 19 and 20, 2000	April 20, 2000	April 24, 2000

Timothy A. Springer, Ph.D.
Manager, Regulatory and Technical Support

4/24/00
DATE

000545

AMENDED

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 454A-102

STUDY DIRECTOR:

Kurt R. Drottar
Senior Biologist

4/26/00
DATE

MANAGEMENT:

Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and
Non-Target Plants

4/26/00
DATE

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SUMMARY

SPONSOR:	3M Corporation
SPONSOR'S REPRESENTATIVE:	Ms. Susan A. Beach
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	454A-102
TEST SUBSTANCE:	PFOS (Perfluorooctane Sulfonic Acid Potassium Salt)
STUDY:	PFOS: A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, 3.3, 5.6, 9.5, 17 and 28 mg a.i./L
TEST DATES:	Experimental Start – February 15, 1999 Biological Termination – February 19, 1999 Experimental Termination – February 19, 1999
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Fathead Minnow (*Pimephales promelas*)	
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. cultures Easton, Maryland 21601	
AGE OF TEST ORGANISMS:	Juveniles	
MEASUREMENTS OF 10 NEGATIVE CONTROL FISH:		
WEIGHT (g):	Mean = 0.36;	Range = 0.21 to 0.49
TOTAL LENGTH (mm):	Mean = 35;	Range = 30 to 38

96-HOUR LC50:	9.5 mg a.i./L
95% CONFIDENCE LIMITS:	8.0 and 11 mg a.i./L
NO MORTALITY CONCENTRATION:	3.3 mg a.i./L
NO-OBSERVED-EFFECT-CONCENTRATION:	3.3 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International Ltd. for 3M Corporation at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from February 15 to February 19, 1999. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 454A-102 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the fathead minnow, *Pimephales promelas*, during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Fathead minnows were exposed to a geometric series of five test concentrations and a negative (dilution water) control. Two replicate test chambers were maintained in each treatment and control group, with 10 fathead minnows in each test chamber for a total of 20 fathead minnows per test concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory range finding toxicity test. Nominal test concentrations selected were 3.6, 5.9, 9.9, 16 and 27 mg active ingredient (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and the control group at the beginning of the test, at approximately 48 hours, and at test termination.

Fathead minnows were indiscriminately assigned to exposure chambers at test initiation. Observations of mortality and other clinical signs of toxicity were made at approximately 2.5, 24, 48, 72 and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups was used to estimate or calculate LC50 values at 24, 48, 72 and 96 hours. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and clinical observation data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "PFOS: A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (*Pimephales promelas*)". The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1075 (1): OECD Guideline for Testing of Chemicals 203: *Fish, Acute Toxicity Test* (2); and ASTM Standard E729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance was received from 3M Corporation on October 29, 1998 and was assigned Wildlife International Ltd. identification number 4675. The test substance was described as a white powder. It was identified as FC-95 from lot number 217 (T-6295). Information provided by the Sponsor indicated a purity of 98.9%, and an expiration data of 2008. The test substance was reanalyzed by the Sponsor and the Certificate of Analysis dated March 9, 2000 indicated a purity of 90.49%. The test substance was stored at ambient room temperature.

Preparation of Test Concentrations

Nominal test concentrations were 3.6, 5.9, 9.9, 16 and 27 mg a.i./L, based on a test substance purity of 90.49%. All materials which came into contact with the test substance during preparation of test concentrations were constructed of plastic or stainless steel. A primary stock solution was prepared in dilution water at a concentration of 27 mg a.i./L. The primary stock solution was mixed with an electric mixer for approximately 22 hours to aid in the solubilization of the test substance. After mixing, the primary stock solution was proportionally diluted with dilution water to prepare the four additional test concentrations. All test solutions appeared clear and colorless.

Test Organism

The fathead minnow, *Pimephales promelas*, was selected as the test species for this study. The fathead minnow is representative of an important group of aquatic vertebrates and was selected for use in the test based upon past history of use in the laboratory. Fathead minnows used in the test were obtained from cultures at Wildlife International Ltd., Easton, Maryland.

Fathead minnows were held at approximately the same temperature as used during the test. The fish were held for approximately 126 days prior to testing. The fish were acclimated to test conditions for approximately 48 hours prior to test initiation. During the holding and acclimation periods, the fish showed no signs of disease or stress. During the 14-day holding period preceding the test, water temperatures ranged from 22.0 to 22.8°C. The pH of the water ranged from 8.2 to 8.5 and dissolved oxygen ranged from 7.7 to 8.4 mg/L. Instrumentation and methods used for water measurements are described in the *Environmental Conditions* section of this report. At test initiation, the fathead minnows were collected from the acclimation tank and transferred to the test chambers.

During the holding period, fathead minnows were fed a commercially-prepared diet. The fish were not fed during the acclimation period (at least 48 hours prior to the test) or during the test.

All fish used in the test were from the same source and year class, and the total length of the longest fish was no more than twice the length of the shortest. The average total length of 10 negative control fish measured at the end of the test was 35 mm with a range of 30 to 38 mm. The average wet weight (blotted dry) of 10 negative control fish at the end of the test was 0.36 grams with a range of 0.21 to 0.49 grams. Loading was 0.24 g fish/L of test water present in the test chambers at any given time.

Test Apparatus

Test chambers were 25-L polyethylene aquaria containing approximately 15 L of test solution. The depth of water in a representative test chamber was approximately 17.6 cm. Test chambers were impartially positioned in an environmental chamber set to maintain a temperature of 22±2°C. The test chambers were labeled with the project number, test concentration and replicate.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity, and pH measurements of the well water during the four-week period immediately preceding the test are presented in Appendix I.

The well water was passed through a sand filter to remove particles greater than approximately 25 μm, and pumped into a 37,800-L storage tank and aerated with spray nozzles. Prior to use, the water again was filtered (0.45 μm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix II.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 391 lux at the surface of the water. Light intensity was measured using a SPER Scientific Ltd. light meter.

Temperature was measured in each test chamber at the beginning of the test and at approximately 24-hour intervals thereafter using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was 22±2°C. Dissolved oxygen and pH measurements were made on water samples from all replicate test chambers of each treatment and control at test initiation and at approximately 24-hour intervals thereafter. Hardness, alkalinity and specific conductance were measured in the dilution water at test initiation.

Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (4).

Observations

Observations were made to determine the number of mortalities. The number of individuals exhibiting clinical signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 2.5, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The 24, 48, 72 and 96-hour LC50 values and the 95% confidence intervals were calculated when possible by probit analysis, the moving average method or binomial probability with non-linear interpolation (5, 6, 7) using the computer software of C.E. Stephan (8). In this study, LC50 values could not be calculated at 24 and 48 hours due to the lack of an adequate concentration-response pattern, however, the probit method was used to evaluate mortality at 72 hours and the moving average method was used to evaluate mortality at 96 hours. The no mortality concentration and NOEC were determined by visual interpretation of the mortality and clinical observation data.

Analytical Chemistry

Water samples were collected at mid-depth from each replicate test chamber of each treatment and control group at the beginning of the test, at 48 hours and at test termination to measure concentrations of the test substance. The samples were collected in plastic (Nalgene®) vials and analyzed as soon as possible without storage. Analytical procedures used in the analysis of the samples are provided in Appendix III.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of PFOS in water samples collected during the test are presented in Table 1 and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 3.6, 5.9, 9.9, 16 and 27 mg a.i./L. Samples collected at test initiation had measured values that ranged from 85 to 117% of nominal values. Measured values for samples taken at 48 hours ranged from 86 to 101% of nominal. Measured values for samples taken at 96 hours ranged from 88 to 98% of nominal. When measured concentrations of the samples analyzed at test initiation, approximately 48 hours and at test termination were averaged, the mean measured concentrations for this study were 3.3, 5.6, 9.5, 17 and 28 mg a.i./L. Mean measured concentrations were used in the estimation or calculation of LC50 values.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH are presented in Table 2. Temperatures were within the 22±2°C range established for the test. Dissolved oxygen concentrations remained ≥ 7.7 mg/L (88% of saturation) throughout the test. Measurements of pH ranged from 8.3 to 8.6 during the test.

Daily observations of mortality and other clinical signs of toxicity observed during the test are shown in Table 3. Fathead minnows in the negative control and the 3.3 mg a.i./L treatment group appeared normal and healthy during the test. After 96-hours of exposure, mortality in the 5.6, 9.5, 17 and 28 mg a.i./L treatment groups was 20, 50, 80 and 100%, respectively. LC50 values and 95% confidence limits at 24, 48, 72 and 96 hours were estimated or calculated from the mortality data, and are shown in Table 4. A graph of the concentration-response curve is presented in Figure 1.

CONCLUSIONS

The 96-hour LC50 value for fathead minnows exposed to PFOS was 9.5 mg a.i./L with 95% confidence limits of 8.0 and 11 mg a.i./L. The 96-hour no mortality concentration and the NOEC were 3.3 mg a.i./L.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine.*

2 **Organisation for Economic Cooperation and Development.** 1993. OECD Guidelines for Testing of Chemicals. *Guideline 203: Fish, Acute Toxicity Test.* Adopted by the Council on 12 July 1992.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

4 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition. American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

5 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

6 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

8 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations*, American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

Table 1

Summary of Analytical Chemistry Data

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Fathead Minnow, *Pimephales promelas*
Dilution Water:	Well Water

Nominal Test Concentration (mg a.i./L)	Replicate	Sampling Time (Hours)	Measured Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Percent of Nominal
Negative Control	A	0	<LOQ[1]	<LOQ	--
	B	0	<LOQ		
	A	48	<LOQ		
	B	48	<LOQ		
	A	96	<LOQ		
	B	96	<LOQ		
3.6	A	0	3.16	3.3	92
	B	0	3.53		
	A	48	3.08		
	B	48	3.22		
	A	96	3.46		
	B	96	3.13		
5.9	A	0	6.05	5.6	95
	B	0	5.07		
	A	48	5.48		
	B	48	5.89		
	A	96	5.70		
	B	96	5.55		
9.9	A	0	8.99	9.5	96
	B	0	9.47		
	A	48	9.88		
	B	48	9.33		
	A	96	9.70		
	B	96	9.52		
16	A	0	18.2	17	106
	B	0	19.3		
	A	48	15.0		
	B	48	15.6		
	A	96	14.8		
	B	96	16.2		
27	A	0	28.5	28	104
	B	0	28.5		
	A	48	27.0		
	B	48	27.8		
	A	96	26.8		
	B	96	26.6		

[1] The limit of quantitation (LOQ) was 0.458 mg a.i./L.

Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Fathead Minnow, *Pimephales promelas*
Dilution Water: Well Water

Mean Measured Test Concentration (mg a.i./L)	Replicate	0 Hour[1]			24 Hours			48 Hours			72 Hours			96 Hours		
		Temp[2] (°C)	DO[3] (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH
Negative Control	A	20.5	8.8	8.6	21.7	7.8	8.5	21.9	8.4	8.4	22.1	8.2	8.4	22.0	7.8	8.3
	B	20.4	8.8	8.6	21.7	7.8	8.5	21.4	8.2	8.4	21.6	8.0	8.4	21.6	7.8	8.4
3.3	A	20.5	8.8	8.6	21.7	7.8	8.5	21.3	8.6	8.5	21.6	8.0	8.4	21.4	7.8	8.4
	B	20.4	8.8	8.6	21.6	7.9	8.5	21.3	8.4	8.5	21.5	8.0	8.4	21.4	7.8	8.4
5.6	A	20.5	8.8	8.6	21.4	7.8	8.5	21.2	8.6	8.4	21.5	8.0	8.4	21.3	7.8	8.4
	B	20.3	8.8	8.6	21.1	7.8	8.5	21.0	8.4	8.4	21.3	8.0	8.4	21.1	7.8	8.4
9.5	A	20.5	9.0	8.5	21.2	7.9	8.5	21.0	8.4	8.4	21.2	8.0	8.4	21.1	7.8	8.4
	B	20.7	9.0	8.5	21.2	7.9	8.5	21.0	8.4	8.5	21.2	8.0	8.4	21.2	7.8	8.4
17	A	21.2	9.0	8.5	21.3	7.8	8.5	21.1	8.6	8.4	21.4	8.0	8.4	21.2	7.9	8.4
	B	21.4	9.0	8.5	21.4	7.9	8.5	21.2	8.5	8.4	21.5	8.0	8.4	21.3	7.8	8.4
28	A	22.2	9.0	8.5	21.4	7.7	8.5	21.3	8.6	8.4	21.6	8.0	8.4	21.5	7.8	8.4
	B	22.3	9.0	8.5	21.7	7.7	8.4	21.7	8.4	8.4	21.9	8.0	8.4	21.8	7.8	8.4

[1] The 0-hour dilution water measurements for hardness, alkalinity and specific conductance were 136 mg/L as $CaCO_3$, 178 mg/L as $CaCO_3$ and 315 µmhos/cm, respectively.
[2] Temperature measured continuously during the test ranged from approximately 20.5 to 22.0°C.
[3] A dissolved oxygen concentration of 5.2 mg/L represents 60% saturation at 22°C in freshwater.

Table 3

Cumulative Percent Mortality and Treatment-Related Effects

Sponsor: 3M Corporation
Test Substance: PFOS
Test Organism: Fathead Minnow, *Pimephales promelas*
Dilution Water: Well Water

Mean Measured Test Concentration (mg a.i./L)	Replicate	No. Exposed	2.5 Hours		24 Hours		48 Hours		72 Hours		Cumulative Percent Mortality	96 Hours		Cumulative Percent Mortality
			No. Dead[1]	Effects[2]	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects		No. Dead	Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		0	10 AN	
3.3	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		0	10 AN	
5.6	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	3	6AN;1E	20
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		1	6AN;3E	
9.5	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	5	5 E	50
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN		5	5 E	
17	A	10	0	10 AN	0	10 AN	0	10 AN	1	2AN;7E	15	9	1 E	80
	B	10	0	10 AN	0	10 AN	0	10 AN	2	3AN;5E		7	3 E	
28	A	10	0	10 AN	0	10 AN	0	10 AN	4	5E;1N	50	10	--	100
	B	10	0	10 AN	0	10 AN	0	10 AN	6	3E;1N		10	--	

[1] Cumulative number of dead fish.

[2] Observed Effects: AN = Appears Normal; E = Erratic swimming; N = Loss of equilibrium.

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Table 4

LC50 Values

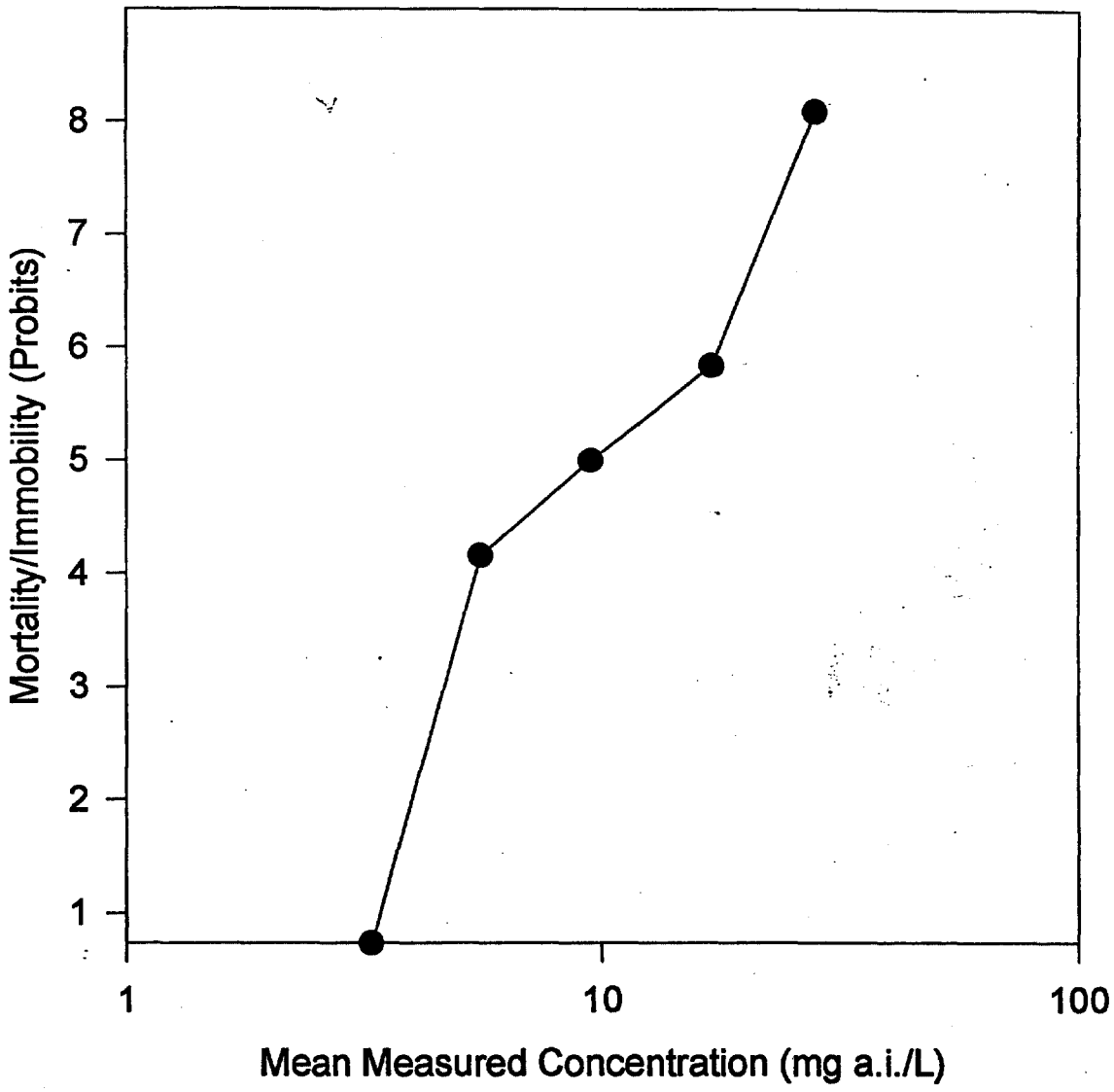
Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Fathead Minnow, *Pimephales promelas*
Dilution Water:	Well Water

Time	LC50 (mg a.i./L)	Lower 95% Confidence Limits (mg a.i./L)	Upper 95% Confidence Limits (mg a.i./L)	Statistical Method
24 Hours	> 28	--[1]	--[1]	Visual Interpretation
48 Hours	> 28	--[1]	--[1]	Visual Interpretation
72 Hours[2]	27	22	41	Probit
96 Hours	9.5	8.0	11	Moving Average

[1] Confidence limits could not be calculated with the mortality data obtained.

[2] The usefulness of this LC50 is questionable because a concentration-effect relationship was not demonstrated over a reasonable range (e.g., <37 to >63) of percent dead.

Figure 1. Concentration-Response Curve (96-Hour Data)



APPENDIX I

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

Sponsor:	3M Corporation
Test Substance:	PFOS
Test Organism:	Fathead Minnow, *Pimephales promelas*
Dilution Water:	Well Water

	Mean	Range
Specific Conductance (μmhos/cm)	311 (N = 4)	310 – 315
Hardness (mg/L as $CaCO_3$)	131 (N = 4)	128 – 136
Alkalinity (mg/L as $CaCO_3$)	177 (N = 4)	176 – 178
pH	8.3 (N = 4)	8.3

APPENDIX II
Analyses of Pesticides, Organics, Metals and Other Inorganics
in Wildlife International Ltd. Well Water[1]

ANALYSIS	MEASURED CONCENTRATION		
Miscellaneous Measurements			
Total Dissolved Solids		286	mg/L
Ammonia Nitrogen	<	0.050	mg/L
Total Organic Carbon[2]	<	1.0	mg/L
Total Cyanide	<	10.0	µg/L
Organochlorines and PCBs			
Aldrin	<	0.005	µg/L
Alpha BHC	<	0.005	µg/L
Beta BHC	<	0.005	µg/L
Delta BHC	<	0.005	µg/L
Gamma BHC (Lindane)	<	0.006	µg/L
Chlordane	<	0.025	µg/L
DDD, pp'	<	0.006	µg/L
DDE, pp'	<	0.005	µg/L
DDT, pp'	<	0.008	µg/L
Dieldrin	<	0.005	µg/L
Endosulfan, A	<	0.005	µg/L
Endosulfan, B	<	0.005	µg/L
Endosulfan Sulfate	<	0.018	µg/L
Endrin	<	0.010	µg/L
Endrin Aldehyde	<	0.005	µg/L
Heptachlor	<	0.005	µg/L
Methoxychlor	<	0.007	µg/L
Heptachlor Epoxide	<	0.005	µg/L
Toxaphene	<	0.500	µg/L
PCB-1016	<	0.260	µg/L
PCB-1221	<	0.260	µg/L
PCB-1232	<	0.260	µg/L
PCB-1242	<	0.720	µg/L
PCB-1248	<	0.720	µg/L
PCB-1254	<	0.720	µg/L
PCB-1260	<	0.720	µg/L
Metals and Other Inorganics			
Aluminum[3]	<	100	µg/L
Arsenic[3]	<	25.0	µg/L
Beryllium[3]	<	0.50	µg/L
Cadmium[3]	<	1.0	µg/L
Calcium[3]		35.0	mg/L
Chromium[3]	<	2.0	µg/L
Cobalt[3]	<	1.0	µg/L
Copper[3]	<	20.0	µg/L
Iron[3]	<	100	µg/L
Lead[3]	<	10.0	µg/L
Magnesium[3]		13.5	mg/L
Manganese[3]	<	1.0	µg/L
Mercury	<	0.20	µg/L
Molybdenum[3]	<	2.0	µg/L
Nickel[3]	<	2.0	µg/L
Potassium[3]		6.62	mg/L
Selenium[3]	<	25.0	µg/L
Silver[3]	<	1.0	µg/L
Sodium[3]		21.3	mg/L
Zinc[3]	<	20.0	µg/L

Analyses performed by QST Environmental, Gainesville, Florida for samples collected on November 3 through November 7, 1997.
Analyses performed by Wildlife International Ltd. for the sample collected on November 5, 1997.
Analyses performed by Wildlife International Ltd. for samples collected on November 5 through 7, 1997.

APPENDIX III

THE ANALYSIS OF PFOS IN FRESHWATER

IN SUPPORT OF

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-102

REPORT APPROVAL

SPONSOR: 3M Corporation

TITLE: PFOS: A 96-Hour Static Acute Toxicity Test with the Fathead Minnow (Pimephales promelas)

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 454A-102

PRINCIPAL INVESTIGATOR:

Raymond L. Van Hoven, Ph.D.
Scientist

4-24-00
DATE

MANAGEMENT:

Willard B. Nixon, Ph.D.
Manager, Analytical Chemistry

4/24/00
DATE

000565

AMENDED

Introduction

Freshwater samples were collected from a static acute aquatic toxicity study designed to determine the effects of PFOS (Perfluorooctane Sulfonic Acid Potassium Salt) to the fathead minnow (Pimephales promelas). This study was conducted by Wildlife International Ltd. and identified as Project No.: 454A-102. The analyses of these water samples were performed at Wildlife International Ltd. using high performance liquid chromatography with mass spectrometric detection (HPLC/MS). Samples were received for analysis on February 15, 17 and 19, 1999 and were analyzed on each sample receipt day.

Test Substance and Internal Standard

The test substance used for the analytical portion of this study was Wildlife International Ltd. identification number 4675. The test substance was used to prepare calibration and matrix fortification samples.

The internal standard was received from 3M Corporation on July 2, 1998 and was assigned Wildlife International Ltd. identification number 4526 upon receipt. The internal standard, a granular material, was identified as: 1H, 1H, 2H, 2H Perfluorooctane Sulfonic Acid, Chemical Abstract Number: 27619-97-2. The standard was stored under ambient conditions.

Analytical Method

The method used for the analysis of the freshwater samples was developed at Wildlife International Ltd. and entitled "Analytical Method for the Determination of PFOS in Freshwater, Saltwater, and Algal Medium". This methodology was included as Appendix II of Wildlife International Ltd. protocol number 454/011299/MVAL/SUB454. It was based upon methodology provided by 3M Corporation.

Samples were diluted in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v) so that they fell within the calibration range of the PFOS methodology.

Concentrations of the PFOS in the standards and samples were determined by reverse-phase high performance liquid chromatography using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph (HPLC) with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-

Elmer TurboIonSpray ion source. HPLC separations were achieved using a Keystone Betasil C_{18} analytical column (100 mm x 2 mm I.D., 3 µm particle size). The instrument parameters are summarized in Table 1. A method flowchart is provided in Figure 1.

Calibration Curve and Limit of Quantitation

Calibration standards of PFOS prepared in a 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v), ranging in concentration from 0.00915 to 0.0457 mg a.i./L were analyzed with the samples. Linear regression equations were generated using peak area response ratios (PFOS : internal standard) versus the respective concentrations of the calibration standards. A typical calibration curve is presented in Figure 2. The concentration of PFOS in the samples was determined by substituting the peak area response ratios into the applicable linear regression equation. Representative ion chromatograms of low and high calibration standards are presented in Figures 3 and 4, respectively.

The method limit of quantitation (LOQ) for these analyses was set at 0.458 mg a.i./L calculated as the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

Matrix Blank and Fortification Samples

Three matrix blank samples were analyzed to determine possible interference. No interferences were observed at or above the LOQ during samples analyses (Table 2). A representative chromatogram of a matrix blank is presented in Figure 5.

Freshwater was fortified at 0.915, 9.15 and 45.7 mg a.i./L and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). Sample concentrations were not corrected for the mean procedural recovery of 97.9%. A representative chromatogram of a matrix fortification is presented in Figure 6.

Example Calculations

Sample number 454A-102-27, nominal concentration of 3.6 mg a.i./L in freshwater.

Initial Volume: 0.250 mL

Final Volume: 25.0 mL

Dilution Factor: 100

PFOS Peak Area: 359685

Internal Standard Peak Area: 415648

Peak Area Ratio: 0.865

Calibration curve equation.

Slope: 0.023

Intercept: 0.062

Curve is linear.

$$\text{PFOS } (\mu\text{g a.i./L}) \text{ at instrument} = \frac{\text{Peak area ratio} - (\text{Y-intercept})}{\text{Slope}}$$

$$\text{PFOS (mg a.i./L) in sample} = \frac{\text{PFOS } (\mu\text{g a.i./L}) \text{ at instrument} \times \text{Dilution Factor}}{1000}$$

Calculated concentration: 3.46 mg a.i./L

Note: manual calculations may differ.

$$\text{Percent of Nominal Conc.} = \frac{\text{PFOS (mg a.i./L) in sample}}{\text{PFOS (mg a.i./L) nominal}} \times 100$$

Calculated recovery: 96.9%

Note: manual calculation may differ.

RESULTS

Sample Analysis

Freshwater samples were collected from the acute toxicity study with the fathead minnow (Pimephales promelas) at test initiation, February 15, 1999 (Hour 0), on February 17, 1999 (Hour 48), and at test termination, February 19, 1999 (Hour 96). The measured concentrations of PFOS in the samples collected at initiation of exposure of the test organisms (Hour 0) ranged from 85.3 to 117% of the nominal concentrations. Samples collected at Hour 48 had a measured concentration range of 86.3 to 101% of nominal values. Samples collected at test termination (Hour 96) had a measured concentration range of 87.6% to 98.3% of nominal values (Table 4). A representative chromatogram of a test sample is shown in Figure 7.

Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer API 100LC Mass Spectrometer equipped with a Perkin-Elmer TurboIonSpray ion source. Operated in selective ion monitoring mode (SIM).
ANALYTICAL COLUMN:	Keystone Betasil C_{18} column (100 mm x 2 mm I.D., 3 µm particle size)
OVEN TEMPERATURE:	30°C
STOP TIME:	10.0 minutes
FLOW RATE:	0.220 mL/minute
MOBILE PHASE:	72.0% Methanol : 28.0% NANOpure® Water containing 0.1% Formic Acid
INJECTION VOLUME:	50.0 µL
PFOS RETENTION TIME:	Approximately 7.1 minutes
INTERNAL STANDARD RETENTION TIME:	Approximately 4.9 minutes
PFOS MONITORED MASS:	498.6 amu
INTERNAL STANDARD MONITORED MASS:	426.7 amu

Table 2

Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample Number (454A-102-)	Type	Measured Concentration of PFOS[1] (mg a.i./L)
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ

[1] The limit of quantitation (LOQ) was 0.458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

Table 3

Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (454A-102-)	Concentrations of PFOS (mg a..i./L)		Percent Recovered
	Fortified	Measured	
MAS-1	0.915	1.03	113
MAS-4	0.915	0.842	92.0
MAS-7	0.915	0.970	106
MAS-2	9.15	8.65	94.5
MAS-5	9.15	8.75	95.6
MAS-8	9.15	8.74	95.5
MAS-3	45.7	48.3	106
MAS-6	45.7	37.6	82.2
MAS-9	45.7	44.0	96.2
		Mean	= 97.9
		Standard Deviation	= 9.12
		CV	= 9.32
		N	= 9

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

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Table 4

Measured Concentrations of PFOS in Freshwater Samples from a Fathead Minnow Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-102-)	Sampling Time (Hours)	PFOS Measured Concentration[1] (mg a.i./L)	Percent of Nominal
0.0	1	0	< LOQ	--
	2	0	< LOQ	--
	13	48	< LOQ	--
	14	48	< LOQ[2]	--
	25	96	< LOQ	--
	26	96	< LOQ	--
3.6	3	0	3.16[2]	88.6
	4	0	3.53	98.9
	15	48	3.08	86.3
	16	48	3.22	90.3
	27	96	3.46	96.9
	28	96	3.13	87.6
5.9	5	0	6.05[2]	102
	6	0	5.07	85.3
	17	48	5.48	92.2
	18	48	5.89	99.0
	29	96	5.70	95.8
	30	96	5.55	93.5
9.9	7	0	8.99[2]	89.3
	8	0	9.47[2]	94.0
	19	48	9.88	97.8
	20	48	9.33	92.8
	31	96	9.70	96.3
	32	96	9.52	94.3

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

[1]The limit of quantitation (LOQ) was 0.458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

[2]Mean result of duplicate redilutions of the original sample.

Duplicate results (in parentheses) for the following 454A-102 sample numbers are: 3 (3.07 and 3.25), 5 (6.11 and 5.99), 7 (9.01 and 8.96), 8 (9.70 and 9.24) and 14 (<0.458 and <0.458). The original results for these samples (4.44, 7.98, 21.2, 2.21, and 0.74, respectively) are not included in the statistical analysis of the data due to suspect dilutions (*e.g.* contamination).

Table 4 (Continued)

Measured Concentrations of PFOS in Freshwater Samples from a Fathead Minnow Static Acute Toxicity Test

Nominal Test Concentration (mg a.i./L)	Sample Number (454A-102-)	Sampling Time (Hours)	PFOS Measured Concentration[1] (mg a.i./L)	Percent of Nominal
16	9	0	18.2	110
	10	0	19.3	117
	21	48	15.0	90.9
	22	48	15.6	94.5
	33	96	14.8	90.2
	34	96	16.2	98.3
27	11	0	28.5	104
	12	0	28.5	104
	23	48	27.0	98.4
	24	48	27.8	101
	35	96	26.8	97.6
	36	96	26.6	97.0

Note: Results and corrections for new test substance purity were generated using MacQuan version 1.5 software and manual calculations. Values have been rounded for reporting purposes.

[1]The limit of quantitation (LOQ) was 0.458 mg a.i./L based upon the product of the lowest calibration standard analyzed (0.00915 mg a.i./L) and the dilution factor of the matrix blank samples (50.0).

[2]Mean result of duplicate redilutions of the original sample.

Duplicate results (in parentheses) for the following 454A-102 sample numbers are: 3 (3.07 and 3.25), 5 (6.11 and 5.99), 7 (9.01 and 8.96), 8 (9.70 and 9.24) and 14 (<0.458 and <0.458). The original results for these samples (4.44, 7.98, 21.2, 2.21, and 0.74, respectively) are not included in the statistical analysis of the data due to suspect dilutions (*e.g.* contamination).

METHOD OUTLINE FOR THE ANALYSIS OF PFOS IN FRESHWATER

Prepare matrix fortification samples by spiking the requisite volume of PFOS stock solutions directly into freshwater using gas-tight syringes and Class A volumetric flasks.

↓

Dilute matrix fortification and test samples into the range of the calibration standards by partially filling Class A volumetric flasks with 50% methanol : 50% NANOpure® water solution containing 0.100 mg 4H PFOS (internal standard)/L and 0.05% formic acid (v/v). Add the appropriate volume of sample and bring the flask to volume with the dilution solvent. Process the matrix blank sample using the same dilution and aliquot volume as for the lowest fortification level. Mix well by several repeat inversions.

↓

Ampulate samples and submit for LCMS analysis.

Figure 1. Analytical method flowchart for the analysis of PFOS in freshwater.

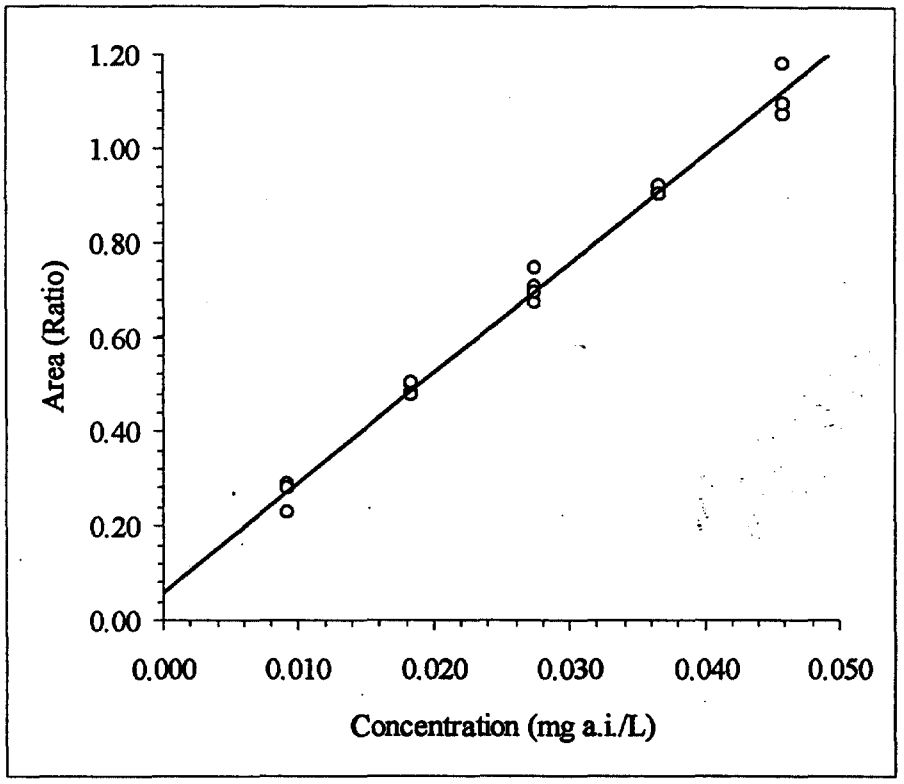

Figure 2. A typical calibration curve for PFOS. Slope = 0.023; Intercept = 0.062; r = 0.996

000576

AMENDED

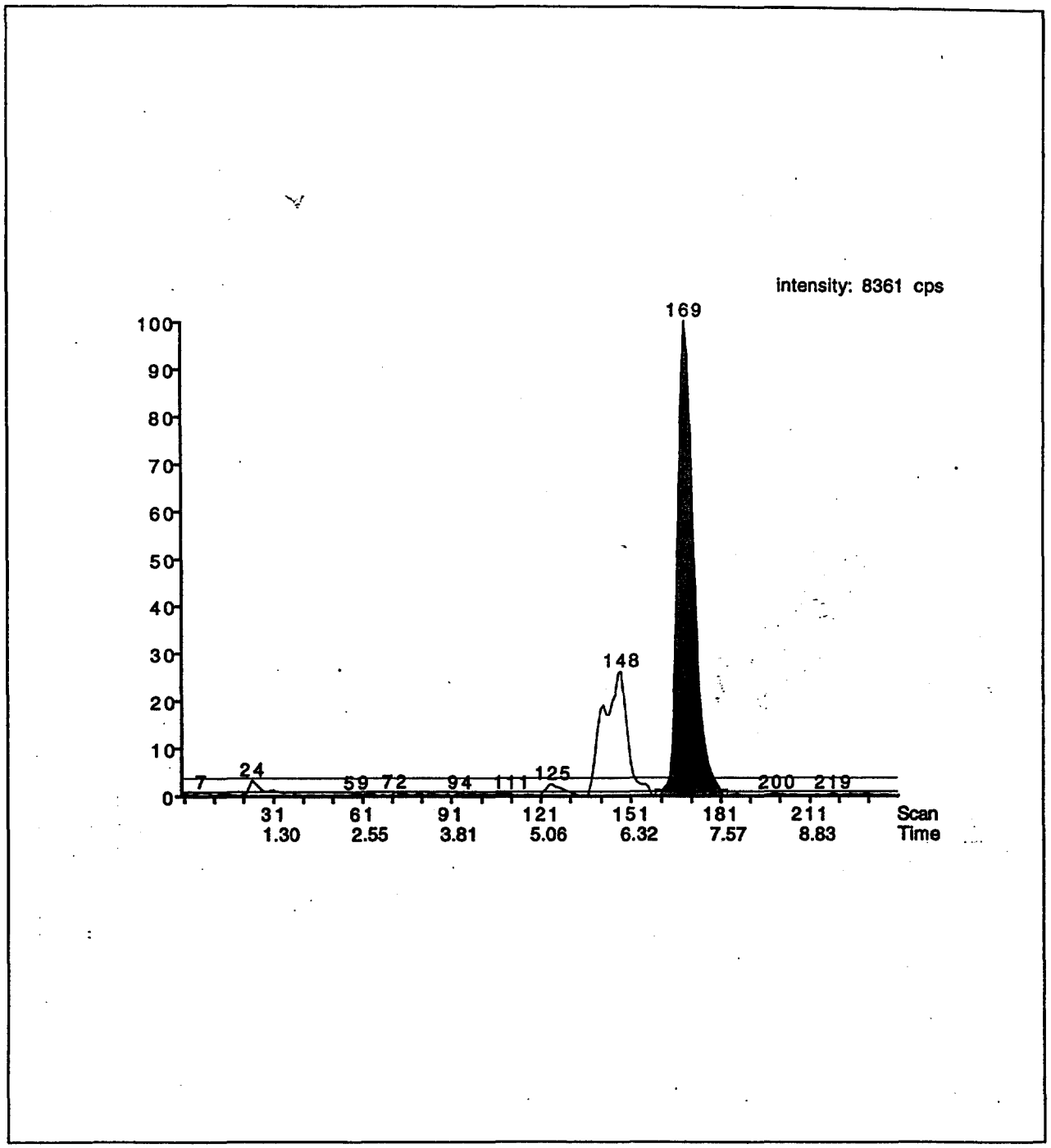

Figure 3. A representative ion chromatogram of a low-level (0.00915 mg a.i./L) PFOS standard.

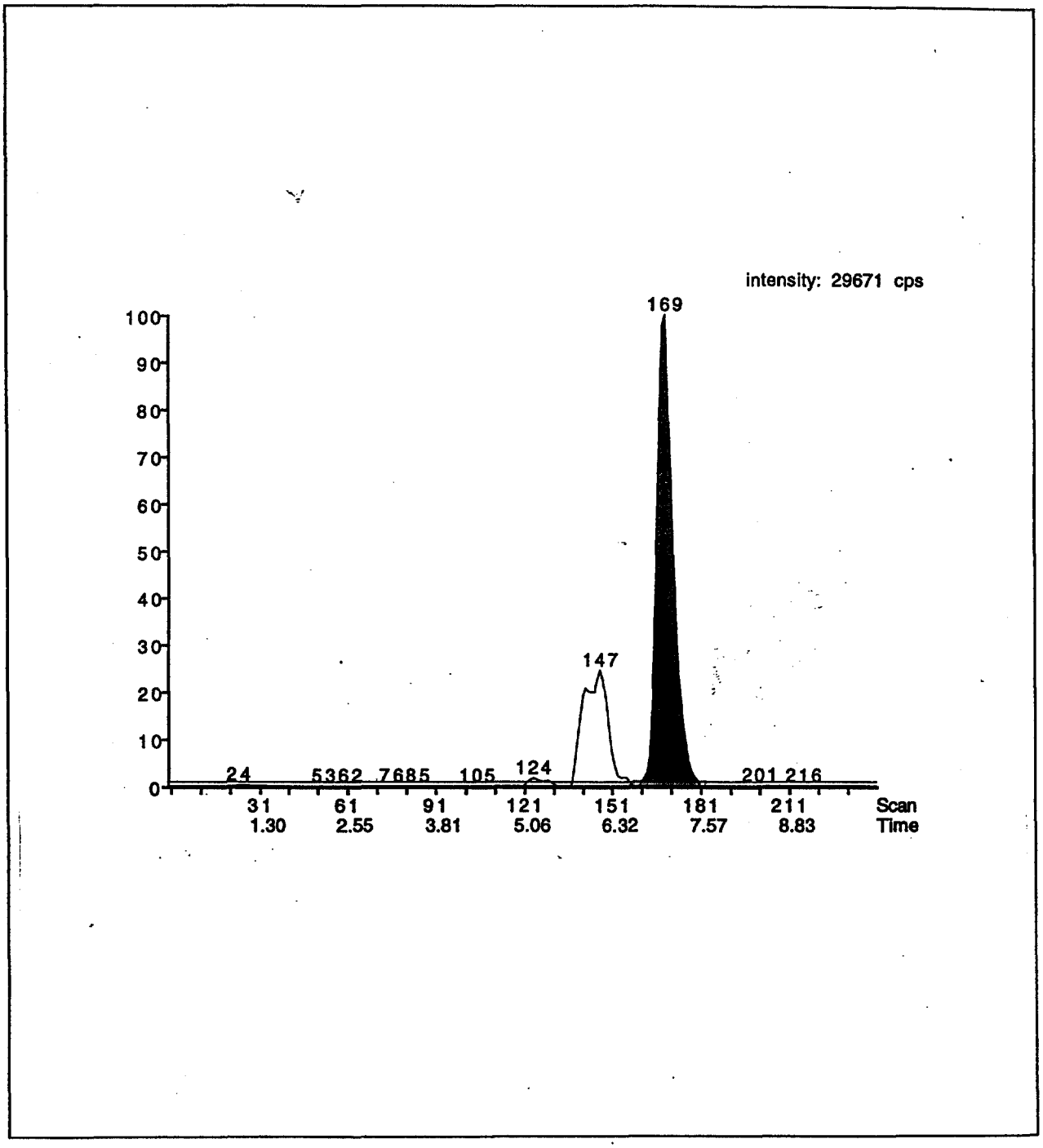

Figure 4. A representative ion chromatogram of a high-level (0.0457 mg a.i./L) PFOS standard.

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AMENDED

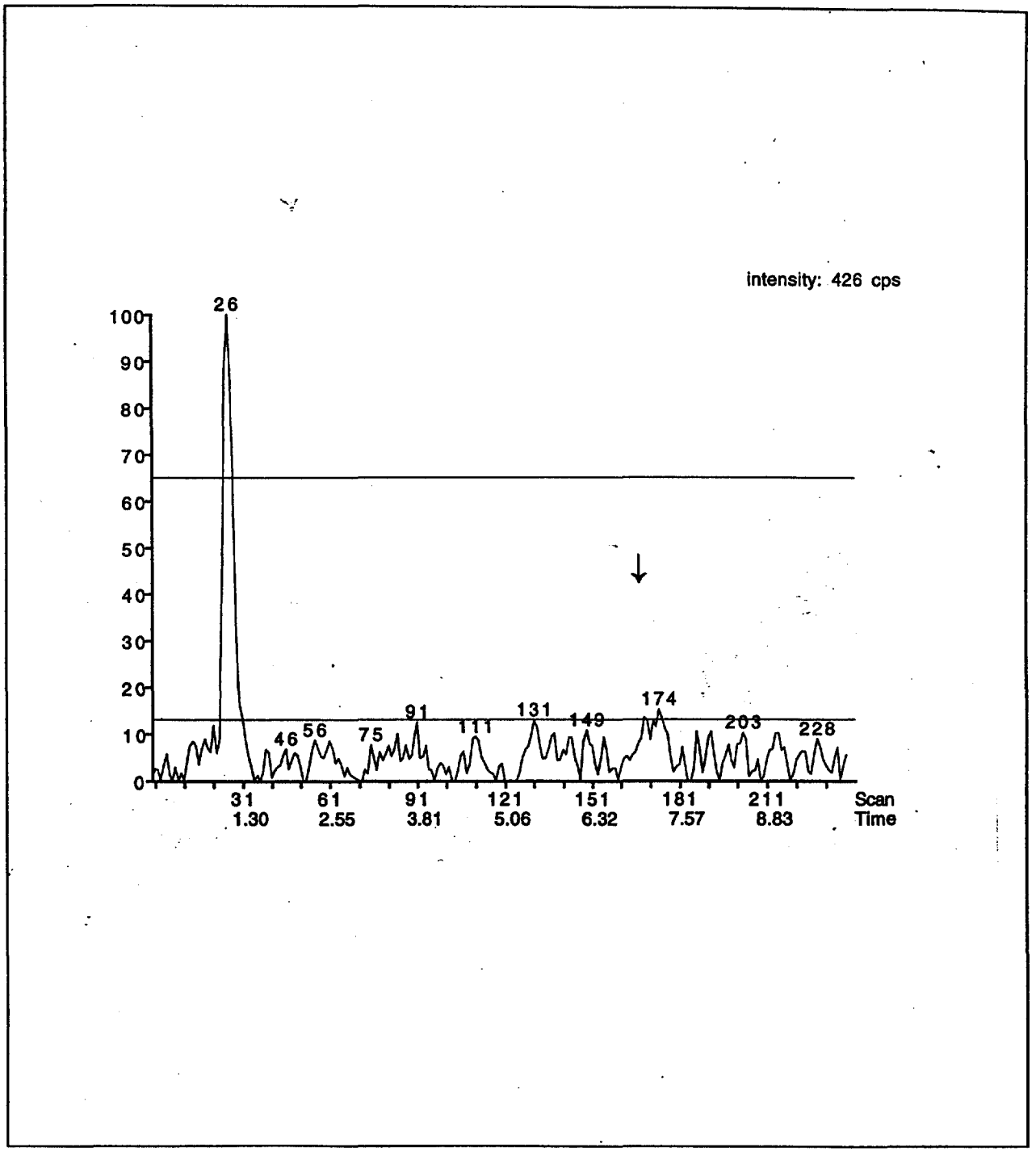

Figure 5. A representative chromatogram of a matrix blank sample (454A-102-MAB-3). The arrow indicates the retention time of PFOS.

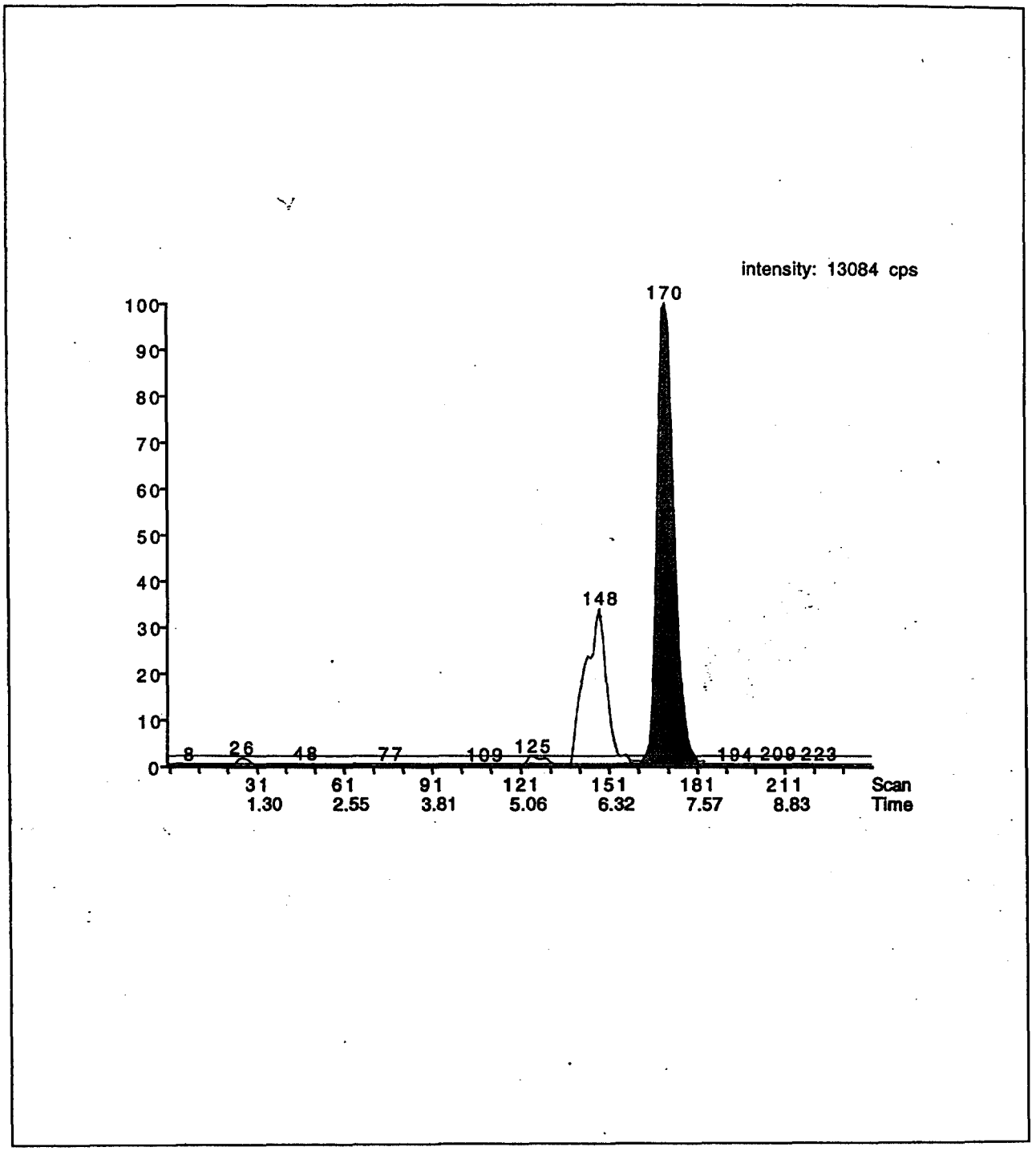

Figure 6. A representative chromatogram of a matrix fortification sample (454A-102-MAS-7).

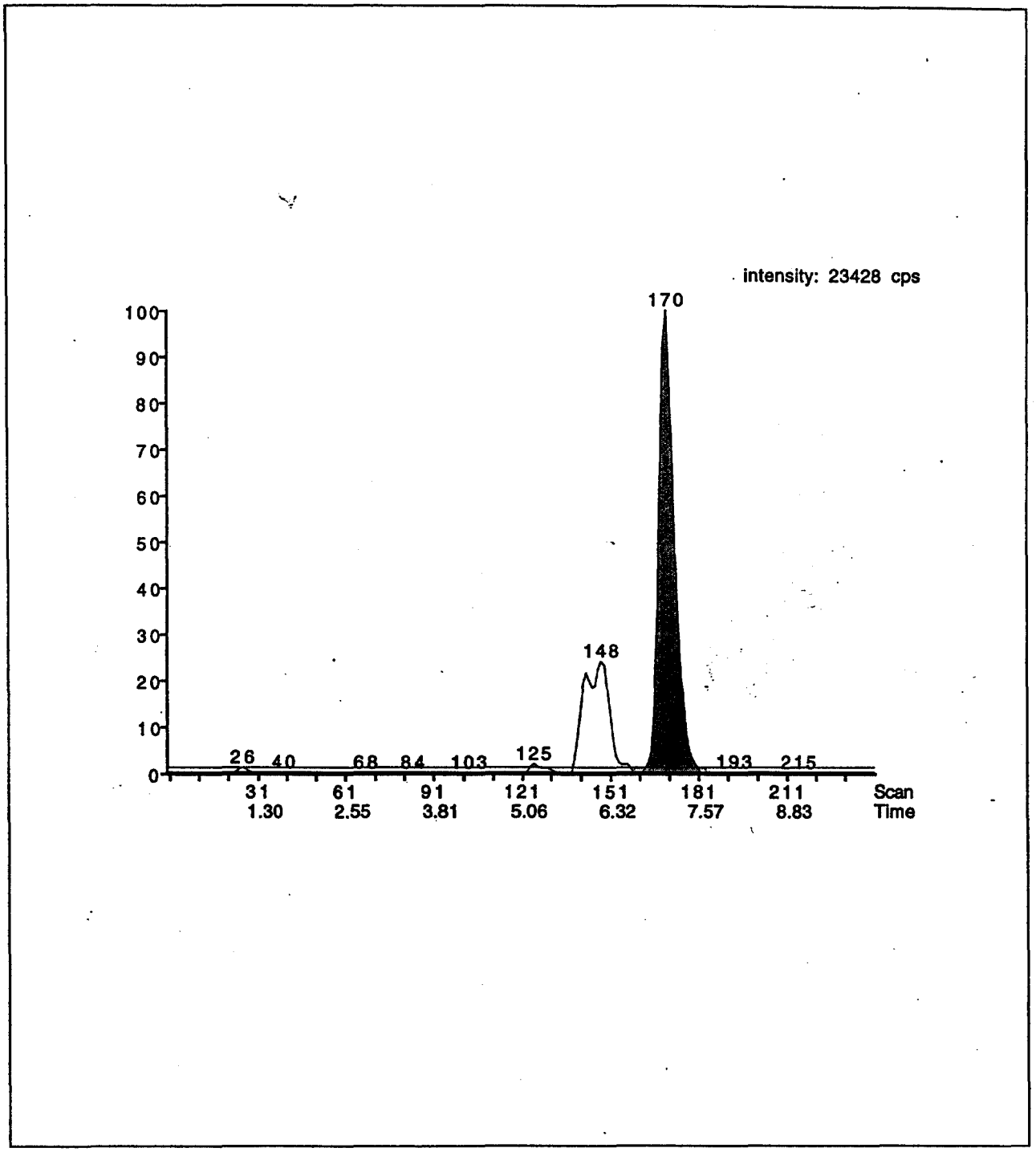

Figure 7. A representative chromatogram of a test sample (454A-102-27).

APPENDIX IV

Changes to Protocol

This study was conducted in accordance with the approved Protocol with the following changes:

1. The protocol was amended to add the proposed experimental start and termination dates, study room, test substance information and test concentrations.
2. The protocol was amended to remove annual feed analysis.
3. The protocol was amended to remove the calculation of an incipient LC50 value.
4. The protocol was amended to specify the analytical method.

APPENDIX V

Personnel Involved in the Study

The following key Wildlife International Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
3. Mark A. Mank, Laboratory Supervisor
4. Timothy Z. Kendall, Laboratory Supervisor
5. Kurt R. Drottar, Senior Biologist
6. Raymond L. VanHoven, Ph.D., Scientist

APPENDIX VI

Report Amendment

1. Original Report: Pages 1-4, 6 and 23

   Amendment: The pages were changed to include the amended report date, revised page numbers, and new signatures and dates due to the addition of the report amendment as Appendix VI.

   Reason: To reflect the issuing of an amended report.

2. Original Report: Page 2

   Amendment: The compliance statement was revised.

   Reason: To clarify how the test substance was characterized.

3. Original Report: Page 9

   Amendment: Information provided by the Sponsor reflecting the reanalysis of the test substance, including the reanalysis date and the purity, was added to the Test Substance section.

   Reason: To reflect the current test substance information provided by the Sponsor.

4. Original Report: Entire report

   Amendment: All test substance concentrations were changed to reflect the purity of the test substance as determined by the Sponsor in a reanalysis of the test substance (FC-95, Lot 217). Test concentrations originally were based on the reported purity of 98.9%. The certificate of analysis dated March 9, 2000 indicated a purity of 90.49%. Therefore, all test substance concentrations, including nominal concentrations, measured concentrations, and LC50 values, were recalculated and reported as mg a.i./L based on the 90.49% purity.

   Reason: To report the results of the test based on the test substance purity of 90.49% at the request of the Sponsor.

APPENDIX VI

-Continued-

Report Amendment

AMENDMENT SIGNATURES:

Kurt R. Drottar
Study Director

4/26/00
DATE

Henry O. Krueger, Ph.D.
Director, Aquatic Toxicology and Non-Target Plants

4/26/00
DATE

REVIEWED BY:

Timothy A. Springer, Ph.D.
Quality Assurance

4/26/00
DATE

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE FATHEAD MINNOW (*Pimephales promelas*)

PROTOCOL NO.: 454/110998/FAT-96H1/SUB454 **AMENDMENT NO.:** 1

SPONSOR: 3M Corporation **PROJECT NO.:** 454A-102

EFFECTIVE DATE: December 21, 1998

AMENDMENT: Page 2

Add: Experimental Start Date: 2/15/99
Experimental Termination Date: 2/19/99
Test Concentrations: Negative Control, 3.9, 6.5, 11, 18 and 30 mg a.i./L
Test Substance No.: 4675

REASON: The above information was not known when the protocol was signed by the Study Director.

AMENDMENT: Test Organism, Page 5

Delete: Feed provided to the fish will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study.

REASON: Historical analyses of Wildlife International Ltd. aquatic feed have shown that no contaminants are present at levels known to be capable of interfering with the study.

AMENDMENT: Data Analysis, Page 8

Change: The LC50 value will be calculated, when possible, using mortality data collected at 24, 48, 72 and 96 hours, as well as the incipient LC50.

To: The LC50 value will be calculated, when possible, using mortality data collected at 24, 48, 72 and 96 hours.

REASON: The incipient LC50 cannot be determined in a 96-hour static acute test.

GLK 2/9/99

AMENDMENT: APPENDIX II, Page 13

Add: Liquid Chromatography Mass Spectrometry (LCMS) Method for the Determination of Perfluorooctane Sulfonic Acid, Potassium Salt (PFOS) in Freshwater, Filtered Saltwater and Algal Medium.

REASON: To add the analytical method to be used in the study.

STUDY DIRECTOR	DATE 2/9/99
LABORATORY MANAGEMENT	DATE 2/9/99
SPONSOR'S REPRESENTATIVE	DATE 2/18/99

PROTOCOL

PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST WITH THE FATHEAD MINNOW (*Pimephales promelas*)

U.S. Environmental Protection Agency
Series 850 - Ecological Effects Test Guidelines
OPPTS Number 850.1075

and

OECD Guideline 203

3M Lab Request No. U2723

Submitted to

3M Corporation
Environmental Laboratory
P.O. Box 33331
St. Paul, Minnesota 55133

WILDLIFE INTERNATIONAL LTD.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

November 9, 1998

000588

PFOS: A 96-HOUR STATIC ACUTE TOXICITY TEST
WITH THE FATHEAD MINNOW (*Pimephales promelas*)

SPONSOR:	3M Corporation Environmental Laboratory P.O. Box 33331 St. Paul, Minnesota 55133
SPONSOR'S REPRESENTATIVE:	Ms. Susan A. Beach
TESTING FACILITY:	Wildlife International Ltd. 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Kurt Drottar Senior Aquatic Biologist
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Director of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: _____ Experimental Termination Date: _____

Project No.: 454A - 102

Test Concentrations: _____

Test Substance No.: _____ Reference Substance No. (if applicable): _____

PROTOCOL APPROVAL

_____ STUDY DIRECTOR	12/14/98 DATE
_____ LABORATORY MANAGEMENT	12/4/98 DATE
_____ SPONSOR'S REPRESENTATIVE	11/30/98 DATE

000589

INTRODUCTION

Wildlife International Ltd. will conduct a static acute toxicity test with the fathead minnow (*Pimephales promelas*) for the Sponsor at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1075 (1); OECD Guideline for Testing of Chemicals 203: *Fish, Acute Toxicity Test* (2); and ASTM Standard E729-88a *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

PURPOSE

The purpose of this study is to determine the acute effects of a test substance on the fathead minnows (*Pimephales promelas*) during a 96-hour exposure period under static test conditions.

EXPERIMENTAL DESIGN

Fathead minnows will be exposed to a geometric series of at least five test concentrations and a negative (dilution water) control for 96 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 fathead minnows in each chamber for a total of 20 fathead minnows per test concentration.

Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target concentrations need not exceed 1000 mg/L or the solubility limit of the test substance in water (whichever is lower). Generally, each test substance concentration used in the definitive test will be
higher concentration unless information concerning the concentration-effe
different dilution factor would be more appropriate. Water samples from
collected at specified intervals for analysis of the test substance. Results
calculate mean measured test concentrations.

To control bias, fathead minnows will be impartially assigned to
initiation. No other potential sources of bias are expected to affect the results
of mortality and other clinical signs will be made throughout the 96-hour test

mortality observed in the treatment groups will be used to calculate, when possible, LC50 values at 24, 48, 72 and 96 hour intervals. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP). The Sponsor is responsible for providing Wildlife International Ltd. written verification that the test substance has been characterized according to GLPs prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International Ltd., it will be noted in the compliance statement of the final report. The attached form **IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR** (Appendix I) is to be used to provide information necessary for GLP compliance.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism directly into dilution water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

Test Organism

The fathead minnow (*Pimephales promelas*) has been selected as the test species for this study. Fathead minnows are representative of an important group of aquatic vertebrates, and have been selected for use in the test based upon past use history in the laboratory. Fish will be from the same source and year class, and the standard length of the longest fish measured will be no more than twice that of the shortest. Fish will weigh between 0.1 g and 3.0 g each and the total weight in each test chamber will not exceed 0.8 grams fish/L of solution. Total lengths and wet weights of the individual fish in one negative control replicate will be measured at the end of the test and will be considered representative of the length and weight of all fish used in the study. Fish will be obtained from a commercial supplier or hatchery, and the identity of the species will be verified by the supplier or by Wildlife International Ltd. personnel using appropriate taxonomic keys, such as Eddy (4).

Fathead minnows will be held for at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test. Any changes in water temperature will not exceed 3 °C per day. Fathead minnows will be held at the test temperature for a minimum of 7 days prior to test initiation. Fathead minnows will not be used in the test if they show signs of disease or stress or if more than 5% die during the 48 hours prior to the test. At test initiation, the fathead minnows will be collected from holding or acclimation tanks and transferred to the test chambers.

During the holding period the test fish will be fed at least once daily. The diet will consist of live or frozen brine shrimp nauplii (*Artemia sp.*), and/or commercial food. Fish will not be fed for at least two days prior to the test or during the test. Feed provided to the fish will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study. Specifications for acceptable levels of contaminants in fish diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

Dilution Water

Water used for the culturing and testing of fathead minnows will be obtained from a well approximately 40 meters deep located on the Wildlife International Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 μm in order to remove fine particles. Water used for culturing and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, μmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the analyses will be summarized in the final report.

Test Apparatus

Test chambers will be 25-L, polyethylene aquaria filled with approximately 15 L of water. Test chambers will be positioned in an environmental chamber or temperature-controlled water bath to maintain a temperature of 22 ± 2°C. Test chambers will be labelled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation, and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be 22 ± 2°C. Temperature will be measured all replicates at the beginning of the test and at approximately 24-hour intervals thereafter using a liquid-in-glass thermometer. Temperature also will be measured with a continuous recorder in one negative control chamber. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in all replicates of the treatment and control groups at test initiation and at approximately 24-hour intervals thereafter using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in all replicates of the treatment and control groups at test initiation and at approximately 24-hour intervals thereafter using a Fisher Accumet Model 915 pH meter, or equivalent. If a treatment group reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken at the next sampling interval, then discontinued.

Hardness, alkalinity, and specific conductance will be measured in the dilution water at test initiation. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent.

Biological Measurements

Observations of mortality and clinical signs of toxicity will be made between 0-24 hours, and at 24, 48, 72 and 96 hours ± 1 hour. Lethality is defined as the lack of visible movement (e.g. lack of fin or opercular movement) in the fish after gentle prodding. All clinical observations including abnormal behavior will be noted.

Sampling for Analytical Measurements

Water samples will be collected from each test chamber at the beginning of the test, during the test, and at the end of the test to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately or placed in an appropriate storage container (e.g., polyethylene or polypropylene bottle) and stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	0 Hours	48 Hours	96 Hours
Control	2	2	2
Solvent Control (if needed)	2	2	2
Level 1-Low Concentration	2	2	2
Level 2	2	2	2
Level 3	2	2	2
Level 4	2	2	2
Level 5-High Concentration	2	2	2
Totals	14	14	14

Total Number of Verification Samples = 42

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, water samples from one or more appropriate test chambers will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

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Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International Ltd. The analytical method used will be based upon methodology provided by the Sponsor and identified in Appendix II. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an LC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The LC50 value will be calculated, when possible, using mortality data collected at 24, 48, 72 and 96 hours, as well as the incipient LC50. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. Test organism history, holding and acclimation records.
5. Stock solution calculation and preparation, if applicable.
6. Observations.
7. Water chemistry results (e.g., alkalinity and hardness).
8. The methods used to analyze test substance concentrations and the results of analytical measurements.
9. Statistical calculations, if applicable.
10. Test conditions (light intensity, photoperiod, etc.).
11. Calculation and preparation of test concentrations.
12. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International Ltd. The report will include, but not be limited to, the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, lengths and weights of a representative group of test organisms, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
14. Statistical methods used to evaluate the data.
15. A graph of the concentration-mortality curve at the end of the test.
16. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
17. The location where raw data and final report are to be stored.
18. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.

19. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (OCDE/GD (92) 32, Environment Monograph No. 45); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). Each study conducted by Wildlife International Ltd. is routinely examined by the Wildlife International Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine.*

2 **Organisation for Economic Cooperation and Development.** 1993. OECD Guidelines for Testing of Chemicals. *Guideline 203: Fish, Acute Toxicity Test.* Adopted by the Council on 12 July 1992.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

4 **Eddy, S.** 1974. *The Freshwater Fishes.* Wm. C. Brown Company Publishers, Dubuque, Iowa.

5 **APHA, AWWA, WPCF.** 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

8 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

9 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

APPENDIX I
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): PFOS (Perfluorooctane Sulfonic Acid Potassium Salt

Reference Standard (if applicable): Analytical Standard: N/A

Internal Standard: 1,1,2,2H,H,H,H Perfluorooctane Sulfonic Acid

Test Substance Sample Code or Batch Number: Lot 217

Test Substance Purity (% Active Ingredient): 98.9 Expiration Date: 2008

II. Test Substance Characterization

Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? Yes ____ No X

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Ambient room temperature

Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? Yes ____ No X

Other pertinent stability information: ____

IV. Test Concentrations:

X Adjust test concentration to 100% a.i. based upon the purity (%) given above.

____ Do not adjust test concentration to 100% a.i. Test the material AS IS.

V. Toxicity Information:

Mammalian: Rat LD50 251 mg/kg Mouse LD50 N/A

Aquatic:

Invertebrate Toxicity (EC/LC50)	Fish Toxicity (LC50)
Daphnia magna: 27 mg/L	Rainbow Trout: 11 mg/L
Daphnia magna: 50 mg/L	Fathead Minnow: 38 mg/L

Other Toxicity Information (including findings of chronic and subchronic tests):

Please see MSDS

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APPENDIX II

Analytical Method to be Provided by Sponsor